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Human Multilineage Progenitor Cell Sensitivity to 4-Hydroperoxycyclophosphamide

Scott D. Rowley, O. Michael Colvin, and Robert K. Stuart

Experimental Hematology Laboratory, Johns Hopkins Oncology Center and Johns Hopkins School of Medicine, Baltimore, Maryland, USA

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Abstract. This institution has documented consistent reconstitution of hematopoiesis in patients treated with marrow lethal chemoradiotherapy who are "rescued" by reinfusion of autologous cryopreserved marrow cells incubated with 4-hydroperoxycyclophosphamide (4-HC) for in vitro purging of occult tumor cells. After 4-HC incubation, the reinfusion marrow cells showed marked reduction in committed progenitor cell (BFU-E, CFU-GM) frequency, and often total absence of detectable progenitors, without significant loss of marrow reconstituting ability. Since BFU-E and CFU-GM assays did not predict marrow reconstituting ability after 4-HC incubation, we sought to determine whether multilineage progenitor cells (CFU-GEMM) might be more resistant to 4-HC incubation and therefore a more reliable predictive assay in this setting. We found that BFU-E, CFU-GM, and CFU-GEMM all show similar dose-related sensitivity to in vitro incubation with 4-HC and do not appear representative of the cell(s) responsible for marrow reconstitution.

Key words: 4-Hydroperoxycyclophosphamide – CFU-GEMM

Successful autologous marrow transplantation is currently limited by residual marrow tumor cells presumed present in certain neoplasms even when a clinical complete remission has been achieved and no detectable tumor persists at the time of marrow harvest. Physical, pharmacologic, and immunologic techniques to separate these clonogenic tumor cells from the marrow hematopoietic stem cells responsible for marrow reconstitution are currently under investigation [1]. Animal experiments from this institution have demonstrated the successful pharmacologic in vitro "purging" of tumor cells from an intentionally contaminated marrow with subsequent rescue of animals following marrow lethal irradiation [2]. These studies employed a cyclophosphamide derivative, 4-hydroperoxycyclophosphamide (4-HC), and demonstrated differing tumor and hemopoietic stem cell sensitivities to high concentrations of cyclophosphamide derivatives not attainable in vivo.

More recently, a phase-I trial of human autologous marrow rescue using cryopreserved 4-HC-incubated marrow has been completed [3]. In this trial, successful hematopoietic reconstitution after marrow lethal chemotherapy with or without total body irradiation was achieved with marrow incubated at 4-HC concentrations up to 120 μg/ml (410 μM), even though virtually all detectable committed progenitor cells (BFU-E, CFU-GM) were eliminated at 4-HC concentrations greater than 80 μg/ml (274 μM). The postincubation survival of committed progenitor cells was not predictive of the marrow repopulating ability of the treated marrow, apparently because committed progenitor cell survival following in vitro incubation with 4-HC does not correlate with the survival of the currently undetectable hematopoietic stem cell. Therefore, successful marrow reconstitution could not be predicted prior to reinfusion of the treated marrow based upon the committed progenitor cell content of the marrow samples.

The multilineage progenitor cell (CFU-GEMM) demonstrates some characteristics possessed by the hematopoietic stem cell including the potential to develop along multiple cell lineages [4], a capacity for self-renewal [5], and a proposed normally quiescent proliferative stage [6]. Although few authors designate the CFU-GEMM as the hematopoietic stem cell responsible for marrow reconstitution [5], many investigators have employed assays of this cell to demonstrate stem cell involvement in various clonal disease states including chronic myelogenous leukemia [7] and polycythemia vera [8]. It is con-

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Address reprint requests to: Dr. Scott D. Rowley, Johns Hopkins Oncology Center, 600 North Wolfe Street, Baltimore, MD 21205, USA.

Table 1. Effect of 4-HC incubation on progenitor cell survival for normal human marrow

4-HC dose (μg/ml)	BFU-E	CFU-GM	CFU-GEMM
0	59.7 ± 6.9[a] (100%)[b]	64.6 ± 5.6 (100%)	8.4 ± 1.9 (100%)
60	22.7 ± 4.4 (42.6%)	35.2 ± 5.7 (54.8%)	2.5 ± 0.9 (31.0%)
100	2.4 ± 1.0 (4.4%)	6.4 ± 2.0 (10.6%)	0.2 ± 0.1 (2.4%)

[a] CFC per 10^5 cells ± SEM. Shown are mean ± SEM of ten separate experiments.
[b] Numbers in parentheses are percent of control (0 μg/ml).

Table 2. Coculture of treated and untreated progenitor cells

Treated[a]: untreated cell ratio	Percent of expected colony number[b]		
	BFU-E	CFU-GM	CFU-GEMM
1.0	93.8 ± 2.0	82.2 ± 5.4	101.8 ± 14.5
2.0	93.0 ± 9.2	93.8 ± 10.2	108.2 ± 24.2

[a] Cells treated at 100 μg/ml 4-HC.
[b] Percent of expected colony number = observed colonies/expected colonies (by summation of colonies formed in separate cultures of treated and untreated cells) × 100. Shown are mean ± SEM values (percent of expected) of four separate coculture experiments.

ceivable therefore that if this cell is directly responsible for marrow reconstitution or representative of the hematopoietic stem cell that is, it would survive incubation with 4-HC in the dose ranges used in the clinical trial described above. This possible decreased sensitivity compared with the previously described dose-dependent sensitivity of the committed progenitor cells should be demonstrable in in vitro cultures. We investigated the sensitivity of CFU-GEMM to 4-HC as an assay of hematopoietic stem cell viability following in vitro incubation with this chemotherapeutic agent.

Materials and methods

4-HC incubation. All in vitro procedures closely followed the incubation procedures employed in the clinical trial cited above. After informed consent was obtained, marrow cells were aspirated from the posterior iliac crests of normal human volunteers. Buffy-coat cells were incubated at 37°C for 30 min in 4-HC freshly dissolved in RPMI 1640 and 20% fetal bovine serum. The final cell concentration was 2×10^7 nucleated cells/ml with a packed red cell volume adjusted to 5%. The incubation was arrested by the addition of chilled medium, and the mononuclear cells (density <1.078) were recovered by Ficoll-Hypaque separation. Cell viability following these steps was determined by trypan-blue dye exclusion.

Cell culture. Mononuclear cells were cultured at 10^5 cells/ml as described by Messner and Fauser [4]. Conditions were 0.9% methylcellulose in Iscove modified Dulbecco medium supplemented by 30% fetal bovine serum, 5% phytohemaglutinin-stimulated leukocyte-conditioned medium, $5 \times 10^{-5} M$ 2-mercaptoethanol, and 1 U/ml sheep plasma erythropoietin (Connaught Laboratories). Treated (100 μg/ml 4-HC) and untreated cells were also cocultured at various seeding densities and in varying ratios of the two samples using the same culture conditions. Quadruplicate 1-ml plates were incubated at 37°C in a 5% CO_2 humidified atmosphere and scored on an inverted microscope for BFU-E, CFU-GM, and CFU-GEMM after 14 days in culture. Selected colonies were aspirated from the plates using a finely drawn Pasteur pipette and stained for cytochemical verification of cellular composition.

Statistics. Statistical analysis was performed using Student's *t*-test, two-tailed.

Results

Treatment of marrow cells from seven normal subjects was carried out at 4-HC concentrations of 0 μg/ml, 60 μg/ml (205 μM), and 100 μg/ml (342 μM) to correspond with levels used in the clinical trial (Table 1). Both the committed cells (BFU-E and CFU-GM) as well as the multilineage cell (CFU-GEMM) demonstrated dose-related sensitivity to 4-HC. At the highest 4-HC concentration, only a rare CFU-GEMM exhibited clonogenic growth potential following treatment. Small numbers of BFU-E and CFU-GM also retained growth potential following exposure to 100 μg/ml of 4-HC. Statistical analysis revealed significant differences ($P < 0.05$) between the number of colonies formed at control and both treatment concentrations for each lineage. The data in fact show a trend (not statistically significant) toward increased, rather than reduced, 4-HC sensitivity of the CFU-GEMM, compared with that of the unipotent progenitor cells. This loss in proliferative capacity following 4-HC exposure was not predicted by loss of trypan-blue exclusion. Cell viability determined by this technique was consistently greater than 90% at each treatment concentration.

Cocultures of treated and control cells were performed to assess the possibility of 4-HC carryover into the culture medium, or the possibility of an accessory cell phenomenon to explain the decreased progenitor cell expression described above (Table 2). The observed numbers of viable progenitor cells forming colonies in the coculture plates were within the range expected for the arithmetic sum of colonies formed from separate cultures of equal numbers of treated and control cells.

Discussion

The relationship of the human CFU-GEMM to the hematopoietic stem cell responsible for marrow repopulation is still under investigation. CFU-GEMM do demonstrate some of the features possessed by the murine spleen colony-forming cell (CFU-S) such as the potential for producing cells that differentiate along multiple lineages including megakaryocytic [9] and lymphocytic [10]. Although its proliferative state is contested [6, 11], it has been described as normally in a quiescent state and actively proliferative in conditions of marrow regeneration such as following bone marrow transplantation [6]. Replating experiments suggest at least a limited capacity for self-renewal [5].

However, the demonstrated toxicity of 4-HC for the CFU-GEMM suggests that this cell is not the hematopoietic stem cell responsible for marrow repopulation, since very few CFU-GEMM are detectable following exposure to 4-HC at concentrations still allowing for successful marrow reconstitution. The sensitivity of the CFU-GEMM to 4-HC is dose-dependent and of somewhat greater magnitude than that of the unipotent progenitor cells.

In our cultures, we did observe very limited survival of all three progenitors, including an occasional CFU-GEMM, at the highest level of 4-HC incubation. This expression (rarely seen in the clinical trial) results from our use of methycellulose culture as opposed to agar culture employed for CFU-C determinations for the clinical trial. Concomitant cultures of 4-HC-exposed normal marrows in methylcellulose and agar demonstrated the greater efficiency of the former medium in permitting clonogenic growth following in vitro incubation with the highest concentrations of 4-HC (data not shown).

The coculture of treated with untreated cells demonstrates that the observed effect of 4-HC in these experiments is upon the progenitor cells (as opposed to an accessory cell) and that this effect occurs during the limited incubation time. The addition of normal marrow cells not incubated with 4-HC did not enhance the growth of 4-HC-treated cells as might be expected if the treated marrow lacked an accessory cell that could be supplied by coculture. This argues against the possibility that exposure of the marrow samples to 4-HC removed a "helper" accessory cell required for in vitro progenitor cell expression. Also, the cocultures demonstrate no significant carryover of 4-HC into the culture medium. (The continuous presence of 4-HC in the culture medium suppresses cell cloning at much lower concentrations of 4-HC [12] than those employed in the limited incubation described here.)

These data do not conclusively demonstrate that the multilineage progenitor cell is not a hematopoietic stem cell with marrow repopulation capacity. Other explanations may be offered. Only limited numbers of CFU-GEMM are detected in in vitro assays, and these may represent only a portion of a heterogeneous progenitor (stem?) cell population. Further modification of culture techniques may allow for detection of additional CFU-GEMM or allow the expression of any cells only sublethally damaged but not capable of proliferation under the described culture conditions. (However, continuing improvement in control CFU-GEMM cloning efficiency in our laboratory has not produced additional colony formation following 4-HC exposure at 100 $\mu g/ml$.)

The present results do question the value of the CFU-GEMM assay as a predictive assay for marrow reconstituting ability following in vitro 4-HC treatment. The dose-dependent sensitivity and the virtual absence of detectable CFU-GEMM at dose levels allowing successful marrow reconstitution precludes the use of this assay as a probe for the quantity of viable stem cells a marrow sample may contain, at least after some in vitro treatments. Other assay systems such as cultures of the recently described "blast cell" with high proliferative capacity [13] may discriminate between committed progenitor and stem cell toxicities following in vitro marrow manipulation and are under active investigation by our laboratory.

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References

1. Kaizer H, Santos GW (1982) Autologous bone marrow transplantation in treatment of leukemia, lymphomas and other cancers. Prog Clin Can 8:31
2. Sharkis SJ, Santos GW, Colvin M (1980) Elimination of acute myelogenous leukemic cells from marrow and tumor suspensions in the rat with 4-hydroperoxycyclophosphamide. Blood 55:521
3. Kaizer H, Stuart RK, Brookmeyer R, Beschorner WE, Braine HG, Burns WH, Fuller DJ, Korbling M, Mangan KF, Saral R, Sensenbrenner L, Shadduck RK, Shende AC, Tutschka PJ, Yeager AM, Zinkham WH, Colvin OM, Santos GW (1985) Autologous bone marrow transplantation (BMT) in acute leukemia: a phase I study of in vitro treatment of marrow with 4-hydroperoxycyclophosphamide (4-HC) to purge tumor cells. Blood (in press)

4. Messner HA, Fauser AA (1980) Culture studies of human pluripotent hemopoietic progenitors. Blut 41:327
5. Ash RC, Detrick RA, Zanjani ED (1981) Studies of human pluripotential hemopoietic stem cells (CFU-GEMM) in vitro. Blood 58:309
6. Fauser AA, Messner HA (1979) Proliferative state of human pluripotent hemopoietic progenitors (CFU-GEMM) in normal individuals and under regenerative conditions after bone marrow transplantation. Blood 54:1197
7. Douer D, Levin AM, Sparkes RS, Fabian I, Sparkes M, Cline MJ, Koeffler HP (1981) Chronic myelocytic leukaemia: a pluripotent haemopoietic cell is involved in the malignant clone. Br J Haematol 49:615
8. Fauser AA, Messner HA (1981) Pluripotent hemopoietic progenitors (CFU-GEMM) in polycythemia vera: analysis of erythropoietin requirement and proliferative activity. Blood 58:1224
9. Fauser AA, Messner HA (1979) Identification of megakaryocytes, macrophages, and eosinophils in colonies of human bone marrow containing neutrophilic granulocytes and erythroblasts. Blood 53:1023
10. Messner HA, Izaguirre CA, Jamal N (1981) Identification of T-lymphocytes in human mixed hemopoietic colonies. Blood 58:402
11. Lu L, Broxmeyer HE, Meyers PA, Moore MAS, Thaler HT (1983) Association of cell cycle expression of Ia-like antigenic determinants on normal human multipotential (CFU-GEMM) and erythroid (BFU-E) progenitor cells with regulation in vitro by acidic isoferritins. Blood 61:250
12. Korbling M, Hess AD, Tutschka PJ, Kaizer H, Colvin OM, Santos GW (1982) 4-hydroperoxycyclophosphamide: a model for eliminating residual human tumour cells and T-lymphocytes from the bone marrow graft. Br J Haematol 52:89
13. Nakahata T, Ogawa M (1982) Hemopoietic colony-forming cells in umbilical cord blood with extensive capability to generate mono- and multipotential hemopoietic progenitors. J Clin Invest 70:1324